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Making the Cut: Innovative Methods for Optimizing Perfusion-Based Migration Assays

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Abstract

Application of fluid shear stress to adherent cells dramatically influences their cytoskeletal makeup and differentially regulates their migratory phenotype. Because cytoskeletal rearrangements are necessary for cell motility and migration, preserving these adaptations under in vitro conditions and in the presence of fluid flow are physiologically essential. With this in mind, parallel plate flow chambers and microchannels are often used to conduct in vitro perfusion experiments. However, both of these systems currently lack capacity to accurately study cell migration in the same location where cells were perfused. The most common perfusion/migration assays involve cell perfusion followed by trypsinization which can compromise adaptive cytoskeletal geometry and lead to misleading phenotypic conclusions. The purpose of this study was to quantitatively highlight some limitations commonly found with currently used cell migration approaches and to introduce two new advances which use additive manufacturing (3D printing) or laser capture microdissection (LCM) technology. The residue-free 3D printed insert allows accurate cell seeding within defined areas, increases cell yield for downstream analyses, and more closely resembles the reported levels of fluid shear stress calculated with computational fluid dynamics as compared to other residue-free cell seeding techniques. The LCM approach uses an ultraviolet laser for “touchless technology” to rapidly and accurately introduce a custom-sized wound area in otherwise inaccessible perfusion microchannels. The wound area introduced by LCM elicits comparable migration characteristics compared to traditional pipette tip-induced injuries. When used in perfusion experiments, both of these newly characterized tools were effective in yielding similar results yet without the limitations of the traditional modalities. These innovative methods provide valuable tools for exploring mechanisms of clinically important

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aspects of cell migration fundamental to the pathogenesis of many flow-mediated disorders and are applicable to other perfusion-based models where migration is of central importance.

Keywords

3D printing; cell migration; computational fluid dynamics; fluid shear stress; laser capture microdissection; smooth muscle cells

CELL migration is involved in many human processes from normal embryogenesis and wound healing to disease pathologies like chronic inflammatory disease and tumorigenesis (1, 2). In cardiovascular tissue, abnormal smooth muscle cell (SMC) migration is a major component of occlusive cardiovascular disease (CVD) (3). Composed of a variety of adherent cell types, the vasculature supplies blood flow to and from working tissues and is inherently exposed to the frictional force of blood in contact with the blood vessel wall known as fluid shear stress. This force is thought to act as a major contributor to a variety of flow-induced vascular remodeling events that contribute to CVD pathogenesis, partially due to its ability to dramatically modulate actin cytoskeletal geometry (4–7). Because adherent cells primarily rely on reorganization of their actin cytoskeleton to translocate and migrate (8, 9), developing physiologically relevant tools for studying cell migration in the context of fluid shear stress is critical.

Manual pipette tip-induced scrape/wound injuries and transwell migration assays dominate the cell migration literature, but the ability to evaluate migration in the same location as perfusion is limited using these traditional approaches. Unlike most treatment methodologies that involve quantification of cell migration after static treatment, exposure of cells to fluid flow requires the addition of a perfusion apparatus which is often accomplished using a parallel plate flow chamber or perfusion microchannel. In microchannels which are inherently inaccessible, migration is often quantified in an alternate location from where the cells originally adhered and were perfused. These adherent cells are either trypsinized or exposed to nonenzymatic cell dissociation solutions which allow cell suspensions to be transferred to secondary vessels (including transwell inserts) to quantify the migratory responses (10). These steps remove the perfused cells from their original orientation, thereby disturbing critical cytoskeletal and focal adhesion adaptations in turn lending unwanted bias and caveats to these approaches.

Cell seeding techniques can also influence the percentage of cells exposed to the reported levels of fluid shear stress. For instance, the use of hydrophobic or diamond pens can be effective at defining cell seeding areas by creating physical barriers impermeable to cell suspensions, but use of these modalities unavoidably elicits unwanted effects on the desired perfusion flow profile due to the residue or scratch imposed on the substrate surface. Obstructing the vessel surface can negatively impact continuous laminar flow by producing barrier-mediated turbulent flow, which can ultimately impact the observed cellular phenotype (11).

Stereolithography (SLA) is the first commercialized additive manufacturing process patented by Charles Hull in 1986 (US Patent 4,575,330) which has become the industry

known as additive manufacturing, rapid prototyping, or 3D printing. This process has become a valuable technology in the design and development of automotive, aerospace, and industrial applications. The SLA process works by curing photopolymer resin layer-by-layer with a focused ultraviolet (UV) laser beam. As with other additive manufacturing processes, no molds or forms are needed. Using fused deposition modeling, a strand of thermoplastic is passed through a heated extruder head which is guided to place a bead of molten plastic in the desired location, where it quickly cools and hardens. Recent years have seen additive manufacturing in biomedical applications such as models for surgical planning, customized implants and prosthetics, and tissue scaffolds (12). In this study, our research group introduces a novel application of additive manufacturing in a laboratory setting which maximizes residue-free cell seeding areas and facilitates long-term cell culture. These attributes make it an ideal tool to be used in studying perfusion-based cell culture models.

Additionally, this study characterizes a newly developed in situ migration technique that uses an UV laser and LCM microscope to provide a touchless technology to rapidly and accurately introduce a custom-sized wound area in perfusion microchannels. We recently introduced the use of LCM technology for conducting wound injuries (13), and herein we further characterize and validate this method for use by comparing it to traditional wound healing methods. A previously characterized touchless technology from Intrexon (laser-enabled analysis and processing; LEAP) (14) performs similarly to our LCM method but is designed to support multiwell platforms and to our knowledge is not used in microchannel or slide-based applications. Our innovative application of LCM elicits comparable wound recovery rates compared to established wound healing injuries and provides a useful solution for accessing otherwise inaccessible microchannels for use in in vitro experimentation. Taken together, these two novel approaches bypass limitations associated with currently used migration protocols and provide more refined modes for analyzing cellular migration in the physiological context of perfused fluid flow.

Materials and Methods

Cell Culture

Rat SMCs (A7r5) were purchased (ATCC; CRL-1444; Manassas, VA) and grown in Dulbecco's modified Eagle's medium (DMEM) containing 0.2% Primocin and 10% fetal bovine serum (Atlanta Biologicals; Flowery Branch, GA) (referred to as "complete media") up to passage 15. Cells were maintained at 37°C and 5% CO₂ in a humidified incubator (ShellLab; Cornelius, OR) and serially passaged 1:2 in tissue culture-treated 75 cm² flasks (USA Scientific; Ocala, FL). For trypsinization, conditioned media was aspirated, flasks were washed with 1× Dulbecco's phosphate buffered saline (DPBS; Thermo Fisher Scientific; Waltham, MA) then cells were incubated for 5 min in 0.25% Trypsin/2.21 mM EDTA in Hank's Balanced Salt Solution (HBSS) without sodium bicarbonate, calcium, or magnesium (Corning; Tewksbury, MA). Cell suspensions (supplemented with complete growth medium) were seeded in appropriate vessels: 60 mm petri dishes, six-well plates (tissue culture (TC)-treated; Corning), and ibidi 6.4 microchannel slides (glass or TC-treated bottoms; ibidi USA; Madison, WI) and allowed to attach/spread for specified times. Images were captured using a Leica DMI4000B inverted fluorescent microscope and CoolSnap HQ2

camera, EVOS FL Auto microscope (Life Technologies), or Carl Zeiss LSM 700 confocal microscope (Carl Zeiss, Germany).

Cell Staining and Fluorescence Microscopy

A7r5 cells were washed in DPBS then stained with complete media containing 5 µg/ml Cell Mask Deep Red Plasma Membrane stain (Molecular Probes; Thermo Fisher Scientific, Waltham, MA). Cells were stained at 37°C for 5 min, after which staining solution was aspirated, cells were washed and fixed with 4% paraformaldehyde for 10 min at 37°C. Cells were washed then counterstained with DPBS containing 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI); 300 nM; Invitrogen; Thermo Fisher Scientific, Pittsburgh, PA) for 5 min at room temperature and washed again and mounted in ibidi Mounting Medium (ibidi USA). Confocal Z-stacks (1 µm optical sections) were collected using a Plan-Apochromat 20×/0.8 M27 objective lens and a 2× optical zoom with a Carl Zeiss LSM 700 confocal microscope (Carl Zeiss) and Zeiss ZEN Black 2011 software. To quantify cell height on a glass substrate, cell height was measured at the center of the nucleus across the *y-z* plane using Zeiss ZEN software. Images were minimally and equally enhanced via histogram using Zeiss ZEN software and Adobe Photoshop CS.

Perfusion Apparati and Computational Fluid Dynamics

Two perfusion apparati were used in this study. Microchannels (ibidi 6.4; Germany) and a parallel plate flow chamber (GlycoTech; Gaithersburg, MD) and peristaltic pump equipped with an in-line flow probe. This maintains a constant flow rate, yielding the desired level of fluid shear stress per manufacturer's guidelines (ibidi, GlycoTech). To compare the wall shear stress (WSS) imposed on adherent cells, three CFD models were developed based on substrate surface and cell profile for the 3D-printed insert and pipette tip method. A model of the flow chamber was developed using SolidWorks (Dassault Systemes, Waltham, MA) with dimensions 2 cm × 1 cm × 0.0254 cm. Inlet and outlet diameters were measured using microcalipers (0.159 cm for the inner diameter of the connecting tubing). The inlet and outlet were extended to limit end-effects in the computational region of interest. To mimic the pipette seeding technique, a cylinder, representing the cells, was included in the model using the cell heights measured at 1 h and 24 h postattachment. The solid model was imported in ANSYS Workbench (ANSYS, Canonsburg, PA), and a 500,000 element mesh was generated using tetrahedral/hybrid shaped elements. A mesh independence test was completed by continuously refining the mesh to include a greater number of elements. By increasing the mesh density from 500,000 elements in 100,000 element increments, the average WSS was able to be compared to determine whether the increased mesh density had an effect on the results. In the confluent cell layer model, the mesh elements were increased from 500,000 to 600,000 with an overall change in the average WSS of 8.77–8.86 dyn/cm². This correlates to approximately a 1% change in average WSS after 500,000 elements. The change in WSS was deemed small enough that the results were mesh independent at 500,000 elements.

The properties of the media used in the lab experiments were calculated using an electromagnetic viscometer (VISCO-lab 4000; Medford, MA) with temperature control jacket set to 37°C. A viscosity value of 0.75 ± 0.01 cP was measured at 37.04 ± 0.08°C then

imported into FLUENT v17.1 (ANSYS). A standard flow rate of 8.6 ml/min was used to determine the inlet velocity which was applied as a blunt profile. The model extension allowed the flow to develop as it reached the computational region of interest. The outlet boundary condition was set as a pressure outlet, while the solver settings were defined as a second order pressure-based solver with steady state flow. The solution converged when the residuals reached a value of 10^{-4} . Postprocessing was completed in CFD-Post (ANSYS).

Additive Manufacturing

The inserts used in this study were made with the SLA process on a Formlabs Form1 + printer. Using 3D computer-aided design software (SolidWorks; Dassault Systemes) solid models were designed then exported as SLA (.stl) files. Surfaces in 3D space were represented by a series of triangles then machine-specific software sliced the model into 100- μ m layers and added support structures to attach the part to the printer's build platform. The build platform was lowered into a tray containing approximately 200 ml resin (methacrylic acid esters, photoinitiators, proprietary pigment, and additive package), and a laser beam was directed by a mirror through the bottom of the translucent resin tray, curing the first layer of the model (or supports). The plate was raised and angled to separate the part from the bottom of the tray and then repositioned to a height of one layer thickness above the previous position to cure the following layer. This process was repeated until completion. Parts were washed in isopropyl alcohol to remove excess resin then postcured at 22°C for 1 h in an UV (405 nm) chamber.

Migration Assays

Two migration assays were used in this study. As previously described for rat SMCs (15), confluent SMCs were quiesced in low (0.2%) serum-containing media for 24 h to prevent potential confounding proliferative effects on migration following both types of injury. All injuries were performed in the center of each confluent SMC layer, and excess cellular debris was immediately washed with $1\times$ DPBS followed by the addition of complete media. Images of each scrape site were captured immediately following (T0) and 16 h (T16) post-washout using quantification methods previously described (15). Briefly, photomicrographs were exported into SCell software (Bellevue, WA), and the annotate/line tool was used to measure five wound widths per image. The average wound width at T16 was divided by the average wound width at T0 which was expressed as percent recovery. For perfusion experiments fold change to static control values (per wounding method) was used to highlight the similar migration effect obtained between different perfusion/wounding methods.

Pipette tip-induced wound healing assay—SMCs were seeded in 3D printed inserts on TC-treated plates and grown to confluence. Sterile P200 pipette tips were used as previously described (15) to manually create tip injuries within vessels.

Laser ablation wound healing assay—SMCs were counted using an automated cell counter (ViCell; Beckman Coulter; Indianapolis, IN) then seeded directly inside TC-treated multichannel slides (ibidi 6.4, Germany) using 30 μ l of cell suspension containing approximately 30,000 cells. Following a brief (30 min) attachment period, each

microchannel reservoir was filled with 60 μ l of complete media and allowed to further attach and spread overnight prior to 24 h quiescence. At desired time points a 5 $\times$ objective and PALM RoboSoftware were used to define the wound area within each microchannel. Next, continued laser pulses were introduced using an UV laser housed on the Carl Zeiss PALM LCM microscope (Carl Zeiss) and the software's AutoLPC (laser pressure catapult) function. Unwanted cells were removed off the slide surface while neighboring cells alongside the cut area were left undisturbed creating a precise 500 μ m denuded zone. MicroBeam cut settings were set to 56% (cut energy) and 84% (focus) while LPC settings were set to an energy delta of 30 and a focus delta of 2. Because each slide inherently possessed a slight curved bottom it was necessary to manually adjust the laser focus between channels to maintain appropriate bright-field focus.

Statistical Analysis

Results are presented as mean $\pm$ standard error of the mean (SEM). Statistical analysis for unpaired groups was performed with Student's two tailed *t* test and comparisons of three or more groups were analyzed with one way ANOVA and Tukey's post hoc test using Sigma Plot v.11.2. A *P* value <0.05 was deemed statistically significant.

Results and Discussion

Trypsinization and Perfusion Dramatically Impact Cell Morphology

To define the morphologic differences between adherent and trypsinized cells in the context of migration studies, rat SMCs were plated to adherence and then individual cohorts were trypsinized from their substrate and analyzed. Phase contrast microscopy (Fig. 1A, B) reveals striking morphologic differences between adherent cells with typical spindle shapes and hill and valley characteristics (16) (Fig. 1A) compared to the same cells 1 min after protease treatment which now appear small and spherical (Fig. 1B). Quantification of trypsinized cell areas using an automated cell analyzer (ViCell) and adherent cell areas using sensitive cell analysis software (SVCe, Bellevue, WA) revealed statistically significant differences in cell areas between these two treatments (Fig. 1C). These observations highlight limitations associated with trypsinization as well as the importance of maintaining an adherent cell type when studying an adherent process like cellular migration.

Independent of protease treatment, cell morphology can be modulated by exposure to fluid shear stress. At the gross level, cells respond to fluid shear stress in a variety of ways. For example, endothelial cells orient parallel while SMCs orient perpendicular to the direction of fluid flow (17, 18). To phenotypically validate our perfusion system, fluorescent imaging of plasma membrane-stained SMCs (Fig. 1D, E) shows the random arrangement of static cells (Fig. 1D) compared to the column-like alignment (Fig. 1E) that a physiologic level of applied fluid shear stress imposes on cytoskeletal rearrangement after 48 h (Fig. 1F). These data provide additional support for preserving the cytoskeletal adaptations that occur in response to this applied force.

Trypsinization methods own an undisputed place in cellular experimental workflows. However, the obvious morphologic changes that promptly occur following protease

treatment argue their utility when studying cellular function following treatments like cellular perfusion which independently modulate cell morphology. Because critical regulators of adherent cell migration, such as cell-to-cell and cell-to-surface contacts as well as intracellular cytoskeletal geometry, will be impacted if cells are forced to dissociate from their substrate, it is important to consider this limitation prior to designing an in vitro perfusion study.

Characterization of 3D Printed Insert Seeding Method

A variety of methodologies exists for seeding cells; however, it is necessary to avoid perturbing the substrate surface while seeding cells in defined residue-free areas. With this in mind, two well-characterized methods for performing these seeding techniques are shown in Figure 2A, B. First, modifying a standard P1000 pipette tip by removing the coned portion of the tip and inverting the flat or wide end of the tip on the substrate surface allows successful cell seeding in this circular area of interest (Fig. 2A). Similarly, surface tension principles permit restricted cell adherence to the outer limits of the cell suspension liquid interface, as illustrated in Figure 2B. Both of these techniques avoid the introduction of unwanted obstructions post-attachment and provide easy, reproducible, and accurate techniques for defining cell attachment area. Importantly, however, neither technique maximizes the perfusion area often defined by the borders of the manufacturer's parallel plate flow chamber gaskets. Moreover, restricted media volume can impact the ability to perform long-term culture using these methods, which hinders flexibility in the experimental design.

In this study, 3D printed inserts prove useful for seeding cells within custom and defined residue-free surface areas which maximizes perfusion area for versatile downstream analyses (Fig. 2C, D). Several inserts made with the FDM process were initially attempted in this study, but two disadvantages became evident. First, the precision of the SLA parts was superior to that of the FDM parts, particularly for the sharp corners of the inserts and the flatness of the face of the insert that interfaces with the slide. Second, the FDM parts are largely porous, as the beads of plastic placed side-by-side will always have some degree of void space. The parts can be sealed by various methods, including treatment with an acetone vapor bath, but this involves an additional process step (19). The SLA inserts performed well in this study without these concerns or with any additional sealing steps. This application illustrates several advantages of additive manufacturing as compared to traditional molding or machining applications: (1) parts are easily customizable with 3D design software; (2) parts can be made inexpensively, even for one-of-a-kind or low volumes; and (3) parts can be made and tested quickly.

Results demonstrate the efficacy of 3D printed inserts for permitting cell attachment and spreading within the entire surface area defined by the dimensions of a parallel plate flow chamber gasket (Fig. 2C, D) and allows long-term culture by increasing the height of the insert wall to accommodate greater solution volumes which translates into greater cell suspension volumes to seed more cells. It is interesting to note that the composition of the 3D insert displays strong autofluorescence with an accompanying halo (Fig. 2C), which is not observed in the pipette tip- or cell suspension-alone photomicrographs (Fig. 2A, B).

Cell Height Impacts Fluid Shear Stress

Most adherent cells attach and spread on the substrate as a function of time, which could influence the magnitude of fluid shear stress or flow profile that is being reported. To demonstrate the impact of cellular height on fluid shear stress, SMCs were seeded then fixed and stained 1 (Fig. 3A) and 24 h (Fig. 3B) postattachment. Z-stacks were imaged using a confocal microscope to accurately measure adherent cell height (Fig. 3C). Results demonstrate that the longer time cells have for attachment, the more flattened and spread out they become. Because non-3D insert, residue-free cell seeding methods are preferred to obstruction-based methods but lack the ability to fully maximize the perfusion area, we performed CFD to illustrate and quantify this theory. Using some of the approaches shown in Figure 2 and the cell heights measured in Figure 3C, three CFD models were tested (Fig. 3D–F). Because the cell area in the 3D insert occupies the entire perfusion area, cell height does not change the applied fluid shear stress. However, the smaller cell areas defined by the standard P1000 pipette tip method showed >6% (1 h attachment; $P < 0.05$) and 5% (24 h attachment; $P < 0.05$) difference in the mean WSS compared to the 3D printed insert model (Fig. 3G). These data demonstrate the importance of maximizing perfusion area and complete cell spreading prior to perfusion onset.

Application of 3D Insert Seeding Method and LCM-Induced Wound Creation for Conducting Perfusion-Based Migration Assays

Microchannels provide a useful alternative to parallel plate flow chambers for performing perfusion experiments, yet studying cell migration in these closed apparatus is limited. We previously demonstrated utility of LCM-induced injuries using glass bottom perfusion microchannels (13); however, characterization of the LCM approach and how it compares to established migration techniques was heretofore untested. In this study, cells were seeded inside TC-treated ibidi 6.4 microchannels and within 3D printed inserts on TC-treated plates and grown to confluence. Cells were then quiesced in low (0.2%) serum containing media for 24 h prior to wounding. For microchannels, cells were quiesced, then an UV laser on a LCM microscope was used to create a defined 500 μm denuded zone within the confluent cell monolayer which recovered in complete media (10% serum) over time (Fig. 4A, C). For pipette tip-induced wounds, 3D inserts were removed, conditioned media was aspirated, then wounds were manually created with pipette tips which similarly recovered over time (Fig. 4B, D). Wound recovery rates were comparable between our LCM technique and the more established pipette tip-induced wound injury under static conditions (Fig. 4E). These data provide solid evidence of the precision and reproducibility of this newly developed, automated LCM migration assay that offers a valuable alternative for studying SMC migration in closed microchannels.

The actin cytoskeleton is a key regulator of cell morphology and adherent cell migration and dramatically responds to alterations in blood flow and fluid shear stress. Therefore, in vitro examination of SMC functionality requires flow/perfusion interventions paired with physiologically relevant migration assays. For these reasons we sought to demonstrate the efficacy of our custom 3D inserts and our LCM technique using a physiologic level of fluid shear stress for conduit arteries (10 dyn/cm^2) (20,21) and a time point previously shown to attenuate SMC migration (4 h) (21).

For these experiments rat SMCs were seeded inside TC-treated ibidi 6.4 microchannels and within 3D printed inserts on TC-treated plates and grown to confluence as explained earlier (Fig. 4). Following quiescence, conditioned media was aspirated and cells were pretreated for 4 h in complete media under static or perfused conditions prior to wounding in each apparatus.

For static pretreatment, complete media was added to confluent monolayers for 4 h prior to wounding as detailed earlier (Fig. 4). Representative images were captured immediately following (Time 0 h) and 16 h postwounding (Time 16 h) in plates (Fig. 5A,C) and in microchannels (Fig. 5E,G).

For perfusion pretreatment, cells were perfused in complete media at 10 dyn/cm^2 for 4 h using a parallel plate flow chamber (GlycoTech) or ibidi 6.4 microchannel (ibidi) connected to a peristaltic pump equipped with an in-line flow probe. This set-up maintained a constant flow rate, yielding the desired level of fluid shear stress per manufacturer's guidelines for each apparatus. After perfusion pretreatment, wounds were created as previously described and representative images were captured in plates (Fig. 5B,D) and in microchannels (Fig. 5F,H).

Our perfusion results agree with previous findings (21) that 4 h of perfusion at 10 dyn/cm^2 is effective at reducing rat SMC migration but demonstrate these findings using both parallel plate flow chambers and microchannels (Fig. 5I). Taken together, these observations demonstrate the efficacy of using both of these tools for studying perfusion-based migration assays.

To our knowledge, the only system capable of carrying out in situ migration assays in microchannels is the electric cell-substrate impedance sensing (ECIS) instrument (Applied Biophysics) (22). Although highly useful and multifunctional, ECIS relies on AC impedance measurements over time, mathematically calculates its components resistance and/or capacitance, and does not permit visual observation of the cells under study including assessment of their morphology or geometry (23). The novel methodology described in this report characterizes an alternative to ECIS that provides users the same ability for accessing and perfusing cells and additionally affords them the ability to visualize wound closure over time and associated cellular morphology/geometric changes. The LEAP instrument also meets this need but is configured to support high throughput multiwell platforms and to our knowledge has not been used in microchannel or slide-based formats (14).

Summary

In this study, we demonstrate a unique and advanced application of additive manufacturing-based 3D printed cell culture inserts that represents a useful tool for cell seeding within custom and defined residue-free surface areas and that can be used in a wide array of cell culture applications for in vitro experimentation. The custom-height design of these inserts deters solution evaporation which facilitates longer incubation times and promotes cell attachment and spreading to better emulate the normal physiological setting of the cells at experiment onset. These characteristics make this product ideal for parallel plate flow

chamber experiments where cell perfusion area is often limited to the manufacturer's defined gasket area which can also be smaller than the growth area of the selected seeding vessel. In addition, in this study we highlight a newly developed, cutting-edge LCM technique for studying SMC migration in otherwise inaccessible locations where cells are traditionally exposed to nonphysiologic experimental influences such as trypsinization and loss of cytoskeletal/focal adhesion geometry. Using these approaches we can accurately and optimally study mechanisms that contribute to flow-mediated cell migration and ensuing tissue remodeling events foundational to occlusive CVD and other migration-based disorders. These findings provide insight into the unique characteristics of and introduce techniques for accurately studying cell migration in the context of in vitro fluid shear stress models.

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Figure 1.

Trypsinization and perfusion dramatically impact cell morphology. (A, B). Phase contrast photomicrographs of adherent rat SMCs before (A) and 1 min after (B) protease treatment. Scale bars are 250 μm . (C) Cell area is significantly smaller in trypsinized SMCs compared to adherent SMCs using SVCell software (adherent) or an automated cell analyzer (trypsinized). (D, E) Fluorescent photomicrographs of plasma membrane stained static (D) and 10 dyn/cm² (E) treated SMCs 48 h post-treatment using perfusion microchannels (ibidi 6.4). Image J analysis reveals perfused cells orient significantly more perpendicular to the direction of fluid flow (arrow) compared to static controls (SC) (F). $n = 10$ cells per group for (C) and (F). * $P < 0.05$ vs. adherent and SC. [Color figure can be viewed at wileyonlinelibrary.com]

Figure 2. Characterization of seeding method using 3D printed inserts. (A–C) Fluorescent photomicrographs of SMCs stained with the live DNA stain Hoescht 33342 and imaged with seeding apparatus in place 1 h postseeding. (A) SMCs seeded inside a modified polypropylene pipette tip; (B) cell suspension bubble; and (C) a newly developed 3D printed insert. (D) Following removal of the 3D printed insert, automated stage enabled stitching of over 60 images using the EVOS FL-Auto microscope reveals a confluent 1.71 cm² monolayer of SMCs. SA: surface area. [Color figure can be viewed at wileyonlinelibrary.com]

Figure 3.

Cell height impacts fluid shear stress. (A–B') Representative confocal images of A7r5 cells plated for 1 h (A, A') or 24 h (B, B') and labeled with DAPI (blue; nuclei) and Cell Mask Deep Red (red; plasma membranes), in x - y plane (A, B) or y - z plane (A', B'). Arrows in A and B represent the cells measured in A' and B', respectively; dotted lines represent the y -axis at which the y - z plane was imaged. Scale bar is 20 μ m. (C) Average cell height at the highest point of the cell was approximated by measuring perinuclear height, as demonstrated in A' and B'. A7r5 cells flatten as they adhere on a glass substrate, decreasing cell height. $n = 14$ cells measured per group. * $P < 0.05$. (D–F) CFD models illustrate the apparent impact cell seeding method has on the applied level of WSS. Simulations of SMCs seeded within

3D printed inserts (D) and within modified polypropylene pipette tips 1 h (E) and 24 h (F) after seeding are used to show seeding methods which do not maximize the perfusion area show more than a 5% increase in average WSS compared to the 3D printed insert cell seeding method. (G) $n = 14$ independent experiments using parameters obtained in Figure 3C. * $P < 0.05$ vs. 3D insert. n.s. = not significant. [Color figure can be viewed at wileyonlinelibrary.com]

Figure 4.

Comparative analysis of LCM versus tip-induced wound healing assays. (A–D) Rat SMCs were seeded inside TC-treated microchannels (A, C) or TC-treated plates (B, D) and grown to confluence then quiesced in low (0.2%) serum containing media for 24 h prior to wound injury. An UV laser on a LCM microscope was used to create a 500 μm denuded zone which recovered over time (A, C). Similarly, manual pipette tip-induced scrape injuries (B, D) recovered over time. Results show the LCM technique performs equally as well to established wound healing techniques (E). $n = 5$ per group. Scale bar is 250 μm /50 pixels. Wound areas were highlighted in green using SCell applied masks. n.s. = not significant. [Color figure can be viewed at wileyonlinelibrary.com]

Figure 5.

Application of insert and LCM in a perfusion study. Rat SMCs were seeded inside inserts (A–D) or microchannels (E–H), grown to confluence, then quiesced in low (0.2%) serum containing media for 24 h prior to pretreatment. Cells were pretreated for 4 h in plates (A–D) or microchannels (E–H) and either remained static (A, C, E, G) or were perfused at 10 dyn/cm² (B, D, F, H) using a peristaltic pump equipped with an in-line flow probe to maintain constant flow rate according to manufacturer's instructions. Following perfusion or static treatment wounds were created using P200 pipette tips (A–D) or LCM injury (E–H)

and phase-contrast photomicrographs of injured cells were captured immediately following injury (Time 0 h) as well as after 16 h (Time 16 h). Migration was measured after 16 h in the presence of normal 10% serum-containing media and expressed as normalized percent wound recovery (T16:T0) then compared to each appropriate apparatus's static control. Results show perfusion pretreatment of 10 dyn/cm² for 4 h significantly attenuates SMC migration after 16 h using a parallel plate flow chamber (PPFC) and the LCM technique (I). $n = 2-3$ independent experiments with 24-62 measurements compared between groups; $*P < 0.05$ vs. SC. Scale bar is 250 μm ; white arrow denotes direction of fluid flow. To clearly see the wound area all images were autocontrasted using Adobe PhotoshopCS4 and cell fronts were traced in white.